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Purification and properties of an oestrogen-stimulated mouse uterine glycoprotein (approx. 70 kDa)

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An oestrogen-induced secretory protein from mouse uterine luminal fluid was purified by CM-Affi-Gel Blue chromatography and reverse-phase h.p.l.c. This protein has an apparent molecular mass of approx. 70 kDa both by SDS/polyacrylamide-gel electrophoresis (with or without 2-mercaptoethanol) and by gel-filtration column chromatography, indicating that it exists as a single-chain polypeptide. Further analysis of the protein revealed that it is highly basic (pI $\geqslant$ 10) and is a glycoprotein. The *N*-terminus appears to be blocked to Edman degradation. The partial amino acid sequence of a fragment was obtained by cleavage with CNBr; no sequence homology was apparent between the analysed fragment and other known sequences. The incorporation of [^{35}S]methionine into uterine proteins *in vitro* revealed that oestrogen treatment of immature mice stimulates both synthesis and secretion of the 70 kDa protein. An enzyme-linked immunosorbent assay with polyclonal antibody was used to determine the tissue distribution of the protein. Tissues such as lung, brain, spleen, muscle, intestine, liver, kidney and ovary of oestrogen-treated mice did not have detectable amounts of the 70 kDa protein. Immunoreactivity was present in uterine and vaginal tissues from oestrogen-treated animals. The 70 kDa protein was not induced by testosterone or progesterone. Although the function of this protein is unknown, it is useful as a marker for the study of oestrogen action in the mammalian uterus as well as regulation of gene expression at the molecular level.

INTRODUCTION

Steroid hormones have been shown to regulate the expression of specific genes in tissues (O'Malley & Means, 1974; Palmiter *et al.*, 1976; Maurer & Gorski, 1977; McKnight, 1978). It is believed that the interaction between a specific steroid-hormone receptor and discrete DNA-binding components near the regulated gene is one of the major steps towards gene activation (Geisse *et al.*, 1982; Govindan *et al.*, 1982; Payvar *et al.*, 1982; Chandler *et al.*, 1983; Compton *et al.*, 1983), yet the control mechanism(s) underlying the orchestration of these multifaceted events remains unsolved.

The mouse uterus is an excellent model system for studies of oestrogen action because it contains oestrogen receptors and depends on oestrogen stimulation for the maintenance of physiological functions. In addition, the availability of many inbred mouse strains and the wide range of their susceptibility to steroid-hormone-induced abnormal development and cancer has been reported (McLachlan *et al.*, 1980). For this reason, oestrogen-responsive proteins in the mouse uterus should provide useful markers for the study of oestrogen action at the molecular level. However, mouse uterine tissue proteins that respond to oestrogen stimulation are present as a minor percentage of the total uterine protein (Katz *et al.*, 1980; Korach *et al.*, 1981; Finlay *et al.*, 1982; Kneifeld *et al.*, 1982), and are therefore difficult to isolate and purify for characterization and for antibody production. The secretions from the uterus of mice (Surani, 1977; Aitken, 1977; Fishel, 1979; Maier *et al.*, 1985) as well as rats (Komm *et al.*, 1985; Kuivanen & DeSombre, 1985) have also been shown to be influenced by oestrogenic hormones. Some of the proteins in the uterine luminal fluid constitute a significant fraction of the total protein and provide a better target for examination. However, the identification and purification of a uterine-specific secretory protein has been hampered by the presence of other protein from serum transudate (Hirsch *et al.*, 1977; Voss & Beato, 1977; Oliphant *et al.*, 1978). In the present study we describe the purification and properties of an oestrogen-stimulated 70 kDa secretory protein from the immature mouse uterus. Also, we demonstrate the hormone-specificity and distribution of this 70 kDa protein in other tissues.

EXPERIMENTAL

Materials

CM-Affi-Gel Blue was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.), horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody and whole goat serum were purchased from Cappel Labora-

Abbreviations used: PBS, phosphate-buffered saline (0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4); TBS, Tris-buffered saline (0.14 M-NaCl/10 mM-Tris/HCl buffer, pH 7.4); e.l.i.s.a., enzyme-linked immunosorbent assay.

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tories (Cochranville, PA, U.S.A.), *o*-dianisidine was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and propan-2-ol was h.p.l.c. grade from Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). All other chemicals were obtained as reagent grade whenever possible. Water was glass-distilled and filtered through a Millipore Norganic cartridge. L-[^{35}S]Methionine (sp. radioactivity 1010 Ci/mmol) was purchased from Amersham–Searle (Arlington Heights, IL, U.S.A.).

Animals

Outbred CD-1 mice [Crl: CD-1 (ICR)BR] from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) were housed under controlled lighting (14 h light/10 h dark) and temperature (21–22 °C) conditions. Germ-free CD-1 mice were also obtained from Charles River Laboratories and maintained in isolation units. Mice were provided with synthetic bedding, fresh water and NIH 31 laboratory mouse chow *ad libitum*. The immature (21-day-old) mice were injected subcutaneously with diethylstilboestrol (Sigma Chemical Co.) in corn oil for 3 consecutive days and killed on day 24 of age by cervical dislocation. The daily dose of diethylstilboestrol was 100 μg/kg body wt. Fluid from the uterine lumen was aspirated with a sterile needle and syringe. The uterine fluid was centrifuged at 60000 *g* for 30 min in a Beckman model TM Airfuge and stored at −70 °C. The effects of testosterone and progesterone were examined by using a regimen similar to that for the diethylstilboestrol treatment with a daily dose of 5 mg/kg body wt.

Assay of uterine protein synthesis

The uterus was removed from the mouse immediately after cervical dislocation and either chopped into small pieces or longitudinally split through the uterine horn to expose the luminal surface. The uterine tissue was rinsed with NIH–H16 low-glucose medium and immediately incubated at 300 mg wet wt./ml of prewarmed NIH–H16 low-glucose/methionine-free medium. The tissue was incubated in the presence of 100 μCi of [^{35}S]methionine/ml under an O_2/CO_2 (19:1) atmosphere at 37 °C for 6 h with constant shaking. At the end of the incubation, tissue and medium were separated by centrifugation at 3000 *g* for 10 min. The supernatant was dialysed against 100 vol. of 125 mM-Tris/HCl buffer, pH 8.0, overnight at 4 °C. The proteins from incubation media were analysed by SDS/12%-(w/v)-polyacrylamide-slab-gel electrophoresis (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue G-250 in methanol/acetic acid/water (5:1:5, by vol.) and subsequently destained with the same solution without dye. The gels were then treated with En^{3}Hance (New England Nuclear, Boston, MA, U.S.A.) and processed for fluorography at −70 °C with Dupont Cronex X-ray (Kodamat X-Omat AR-5) film with a Dupont Quanta III intensifying screen. The protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Isoelectric focusing

Isoelectric focusing was carried out in thin-layer LKB Ampholine polyacrylamide-gel plates in the pH range of 3.5–9.5 in an LKB Multiphor unit. Focusing was carried out for 80 min at a constant power of 10W with an initial current of 17 mA and a voltage maximum of 1.2 kV.

Purification of the 70 kDa protein

CM-Affi-Gel Blue was obtained from Bio-Rad Laboratories and treated according to the specifications of the supplier. Uterine luminal fluid was diluted with 10 mM-sodium phosphate buffer, pH 8.0, containing 0.14 M-NaCl to a protein concentration of 1 mg/ml and applied to a 1.5 cm × 15 cm CM-Affi-Gel Blue column equilibrated with the same buffer. The column was washed with the same buffer until the A_{280} returned to the baseline. Protein samples bound to the column were eluted first with 10 mM-phosphate buffer, pH 8.0, containing 0.5 M-NaCl and then with 10 mM-phosphate buffer, pH 8.0, containing 1.4 M-NaCl. The salt was removed by dialysing the samples against several changes of 10 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA at 4 °C. After freeze-drying, protein samples were redissolved in a small quantity of deionized water and analysed by SDS/polyacrylamide-gel electrophoresis. The partially purified 70 kDa protein from the CM-Affi-Gel Blue column was further purified on a Waters h.p.l.c. system equipped with a U6K injector and a Bio-Rad RP-304 C4 (5 μm particle size, 33 nm pore size) column. The protein was dissolved in aq. 0.1% (v/v) trifluoroacetic acid, applied to the column and eluted with a linear gradient. The initial solvent condition of 25% (v/v) propan-2-ol in aq. 0.1% trifluoroacetic acid was increased to 67% (v/v) propan-2-ol in aq. 0.1% trifluoroacetic acid over 20 min, and this was then maintained for 40 min. Flow rate (0.5 ml/min) and solvent parameters were programmed with a Waters 720 system controller. The eluent was monitored at 280 nm with a Waters 440 u.v. detector and relative peak areas were determined with a Waters 730 data module. Fractions were collected in borosilicate-glass tubes and evaporated to dryness in a Speed Vac concentrator system (model SVS 100 H; Savant Instruments, Hicksville, NY, U.S.A.).

Gel filtration

A 1 ml portion of uterine luminal fluid was applied to a column (0.9 cm × 58 cm) of Sephadex G-150 (40–120 mesh; Pharmacia, Piscataway, NJ, U.S.A.) equilibrated with PBS; 0.6 ml fractions were collected and freeze-dried. Fractions were analysed on SDS/polyacrylamide-gel electrophoresis without the presence of 2-mercaptoethanol.

Amino acid and carbohydrate analysis

Total neutral sugar content was determined by the phenol/H_2SO_4 method (Dubois *et al.*, 1956), with mannose as a standard. Sialic acid was determined by the method of Aminoff (1961), after hydrolysis of the glycoprotein in 50 mM-H_2SO_4 for 1 h at 80 °C. The glycoprotein was hydrolysed in 0.5 M-H_2SO_4 for 5 h at 100 °C under vacuum for determination of individual neutral sugars. Hydrolysates were diluted with water and applied to coupled columns of Dowex 50 X4 (200–400 mesh; H^+ form) and Dowex 1 X8 (200–400 mesh; formate form); the columns were then washed with 4–5 column volumes of water. The isolated neutral sugars were converted into alditol acetate derivatives and analysed by g.l.c. (Mohapatra *et al.*, 1983; Bhattacharyya *et al.*, 1984). The glycoprotein was hydrolysed under vacuum in 4 M-HCl for 6 h at 100 °C for the determination of hexosamines. Hexosamines in the hydrolysate

were determined on a Glenco amino acid analyser as described previously (Bhattacharyya *et al.*, 1984). Amino acid analyses were performed on the amino acid analyser after hydrolysis of the glycoprotein in 6 M-HCl for 20 h at 105 °C in an evacuated sealed tube.

Amino acid sequence

Automated sequence analysis of 1–5 nmol of the 70 kDa protein or its CNBr-cleavage derivatives was performed with a gas-phase micro Sequenator (model 740 A; Applied Biosystems, Foster City, CA, U.S.A.).

CNBr cleavage

Cleavage of the 70 kDa protein by CNBr (Gross, 1967) was performed by dissolving 100–300 μg of the h.p.l.c.-purified freeze-dried protein in 0.5 ml of 70% (v/v) formic acid. Approx. 1 mg of crystalline CNBr (Sigma Chemical Co.) was added and the reaction allowed to proceed for 24 h at 22 °C under N_2. Then 10 vol. of deionized water was added and the sample was freeze-dried; the same volume of water was added to the powder and the sample was freeze-dried again. The powder was finally dissolved in aq. 0.1% trifluoroacetic acid and eluted on an h.p.l.c. system similar to that described above except that the linear gradient was 20–50% (v/v) acetonitrile in aq. 0.1% trifluoroacetic acid over a 25 min period.

Gel electrophoresis and electroblotting

The electrophoretic transfer of protein from SDS/polyacrylamide gel on to nitrocellulose paper was performed in accordance with Towbin *et al.* (1979) with a constant current of 200 mA at 4 °C for 18 h in a Bio-Rad Transphore apparatus. Protein blots were stained with India ink (Hancock & Tsang, 1983) for location of the protein transferred or immunostained (Burkhart *et al.*, 1985) for the demonstration of antibody specificity.

Antibody production

Antisera to the h.p.l.c.-purified 70 kDa protein were made by injecting 400 μg of protein emulsified with Freund's complete adjuvant at multiple sites on the dorsal surface of the rabbit. Booster injections of 200 μg of 70 kDa protein emulsified with Freund's incomplete adjuvant were administered at 3, 7 and 10 weeks after the first injection. Blood was collected 1 week after each injection. All three rabbits produced immunoprecipitable antibody against 70 kDa protein when examined on an Ouchterlony immunodiffusion plate. Antiserum from one rabbit (8345) demonstrated the greatest affinity for 70 kDa protein and was used for all experiments. The IgG fraction used in immunostaining experiments was precipitated from the antiserum at 18% (w/v) Na_2SO_4. The precipitate was collected by centrifugation at 300 ***g*** for 30 min at room temperature, redissolved in a small volume of PBS and then dialysed extensively against the same buffer. The protein concentration was adjusted to 1 mg/ml and the solution was stored at −70 °C until use.

Immunostaining

The IgG fraction from the rabbit anti-(70 kDa protein) serum was diluted 200-fold with 4% (v/v) normal goat serum in TBS and used as the first antibody. The final concentration of IgG ranged from 10 to 100 μg/ml. The second antibody [peroxidase-conjugated goat anti-(rabbit IgG) antibody] was absorbed with 20% (v/v) mouse erythrocytes (prewashed with TBS) at 2 h at 4 °C after reconstitution. The erythrocytes were removed by centrifugation and the antibody was diluted 1:150 with 4% normal goat serum in TBS before use. Immunodetection of 70 kDa protein on the nitrocellulose paper was performed as follows. The nitrocellulose paper was blocked in 4% (w/v) bovine serum albumin in TBS for 2 h at 42 °C with gentle agitation. The paper was washed in TBS for 30 min at room temperature. The blots were then incubated with the first antibody for 2 h at 37 °C. After being washed in TBS for 40 min with five or six changes of buffer, the blots were blocked with 4% bovine serum albumin one more time before incubation with the peroxidase-conjugated second antibody for 1 h at 22 °C. After the second incubation, the papers were washed again in TBS for 40 min with five or six changes of buffer. The development of the colour was accomplished by addition of 0.0025% *o*-dianisidine and 0.01% H_2O_2 in TBS buffer for 20 min; the papers were then washed in distilled water and blotted dry.

Quantification by e.l.i.s.a.

E.l.i.s.a. was performed in accordance with Engvall (1980). The antiserum to the 70 kDa protein was used in a 1:2000 dilution (first antibody) and the horseradish-peroxidase-conjugated anti-(rabbit IgG) antibody (second antibody) was used in a 1:4000 dilution in PBS containing 0.05% Tween 20. Tissue was homogenized in PBS and centrifuged at 16000 ***g*** for 30 min. The supernatant fractions were collected and assayed quantitatively for the 70 kDa protein by e.l.i.s.a. Samples (50 μl) in 50 mM-sodium carbonate buffer, pH 9.6, were applied to the wells and incubated for 16 h at 4 °C. The wells were blocked with 0.1% gelatin in PBS for 1 h, and incubated with first antibody for 2 h and then second antibody for 1 h, at room temperature. Between each incubation, wells were washed with PBS containing 0.05% Tween 20 five times. The peroxidase reaction was performed by addition of 0.1% *o*-phenylenediamine and 0.067% H_2O_2 in 20 mM-citric acid/50 mM-sodium phosphate buffer, pH 5.0. After a 10 min reaction period, the absorbance was read at 492 nm in a Titertek Multiskan instrument (Flow Laboratories).

Plasminogen activator determination

Samples of uterine luminal fluid and purified 70 kDa uterine secretory protein were assayed for proteinase and plasminogen activator activities by SDS/polyacrylamide-gel electrophoresis with co-polymerized substrates according to Heussen & Dowdle (1980).

Computer search

Sequence homology of the 70 kDa protein with other protein was screened through Protein Sequence Data Bases (Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Georgetown University, Washington, DC, U.S.A.).

RESULTS

Oestrogen-stimulated synthesis

About 0.1–0.2 ml of fluid can be collected from the uterine lumen of an oestrogen-stimulated immature mouse. At least 20 bands were detected by staining with Coomassie Blue after separation of the luminal-fluid

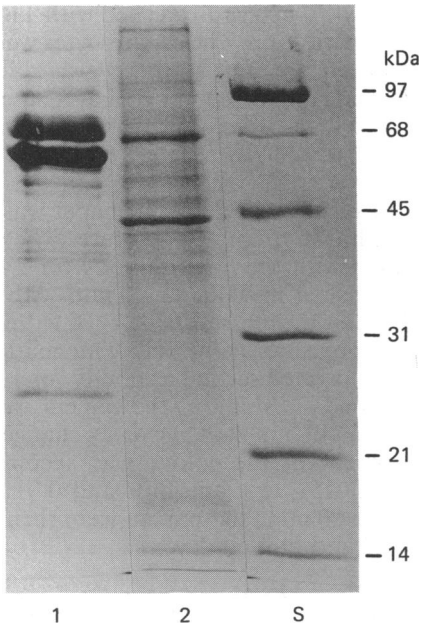

Fig. 1. SDS/polyacrylamide-gel-electrophoretic analysis of proteins from uterine luminal fluid (lane 1) and uterine tissue homogenate lane (2)

Total mouse uterine tissue protein was prepared from PBS-washed oestrogen-stimulated immature mouse uterus. The tissue was homogenized in 125 mM-Tris/HCl buffer, pH 7.0, containing 1% SDS and 0.5% dithiothreitol. The homogenate was centrifuged at 60000 *g* for 30 min. Proteins in the supernatant were analysed by SDS-polyacrylamide-gel electrophoresis as described in the Experimental section. A 150 μg portion of proteins was loaded for each sample and the proteins were localized by Coomassie Blue staining. Phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soya-bean trypsin inhibitor (21 kDa) and lysozyme (14 kDa) were used as molecular-mass standards (lane S).

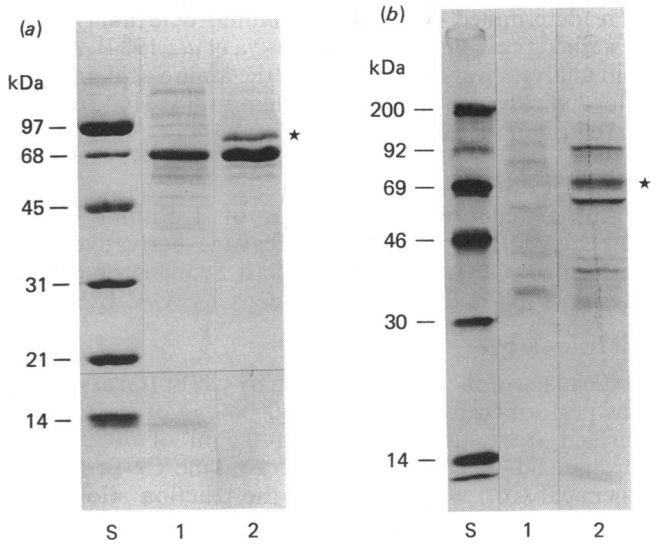

Fig. 2. SDS/polyacrylamide-gel electrophoretic analysis of proteins secreted into incubation medium by immature mouse uteri

The 21-day-old control or diethylstilboestrol-stimulated immature mouse uteri were incubated with the presence of [^{35}S]methionine for 6 h as described in the Experimental section. Protein secreted into incubation medium was partially purified through CM-Affi-Gel Blue column chromatography: 0.5 M-NaCl eluted proteins were analysed by SDS/12%-polyacrylamide-gel electrophoresis. (*a*) Coomassie Blue stain of 80 μg of protein from control (lane 1) or diethylstilboestrol-stimulated (lane 2) mouse uteri. (*b*) Fluorogram of equal amounts of radioactivity from [^{35}S]methionine-labelled protein from control (lane 1) or diethylstilboestrol-stimulated (lane 2) mouse uteri. Molecular-mass standards are shown in lane S in each case.

proteins by SDS/polyacrylamide-gel electrophoresis. Among them, two predominant proteins appeared at the positions of approx. 70 and 63 kDa (Fig. 1, lane 1). Total protein from oestrogen-stimulated mouse uterine tissue did not show such predominant bands on SDS/polyacrylamide-gel electrophoresis (Fig. 1, lane 2).

Synthesis of mouse uterine secretory protein was examined. Immature uteri obtained from control mice and mice subjected to oestrogen stimulation for 3 days were incubated *in vitro* in the presence of [^{35}S]methionine for 6 h, and the medium was separated from the tissue at the end of incubation. Total protein released into the medium was fractionated by CM-Affi-Gel Blue column chromatography and examined by SDS/polyacrylamide-gel electrophoresis. The 0.5 M-NaCl-eluted protein pattern was different between control and oestrogen-stimulated immature uteri (Fig. 2*a*). The 70 kDa protein was significantly increased in oestrogen-stimulated uterine incubation medium. Fluorograms of the gels revealed that the 70 kDa protein was one of the major proteins synthesized in oestrogen-stimulated immature uteri (Fig. 2*b*).

Purification

The 70 kDa protein comprised about 10–20% of the total protein in the uterine luminal fluid, as revealed by Coomassie Blue staining of SDS/polyacrylamide gels after electrophoresis. We have used the CM-Affi-Gel Blue to separate any serum albumin from other proteins since serum proteins may be the major transudate in uterine fluid. The uterine luminal fluid was diluted with 10 mM-phosphate buffer, pH 8.0, containing 0.14 M-NaCl to 1 mg of protein/ml and passed through a CM-Affi-Gel blue column, and the bound protein was eluted with 10 mM-phosphate buffer, pH 8.0, containing 0.5 M-NaCl and then with 10 mM-phosphate buffer, pH 8.0, containing 1.4 M-NaCl. Albumin was tightly bound to the CM-Affi-Gel Blue resin and eluted with high salt concentration (1.4 mM-NaCl). Transferrin, another major serum protein (approx. 76 kDa), was not bound to the resin and appeared in the void fraction. The 70 kDa protein in the uterine luminal fluid was bound to the CM-Affi-Gel Blue resin and eluted by 0.5 M-NaCl (Fig. 3*b*, lane 3). This chromatographic step thus removed the major potential serum contaminants from the 70 kDa protein and also partially purified the 70 kDa protein from total uterine luminal-fluid protein. The 0.5 M-NaCl fractions from the CM-Affi-Gel Blue column

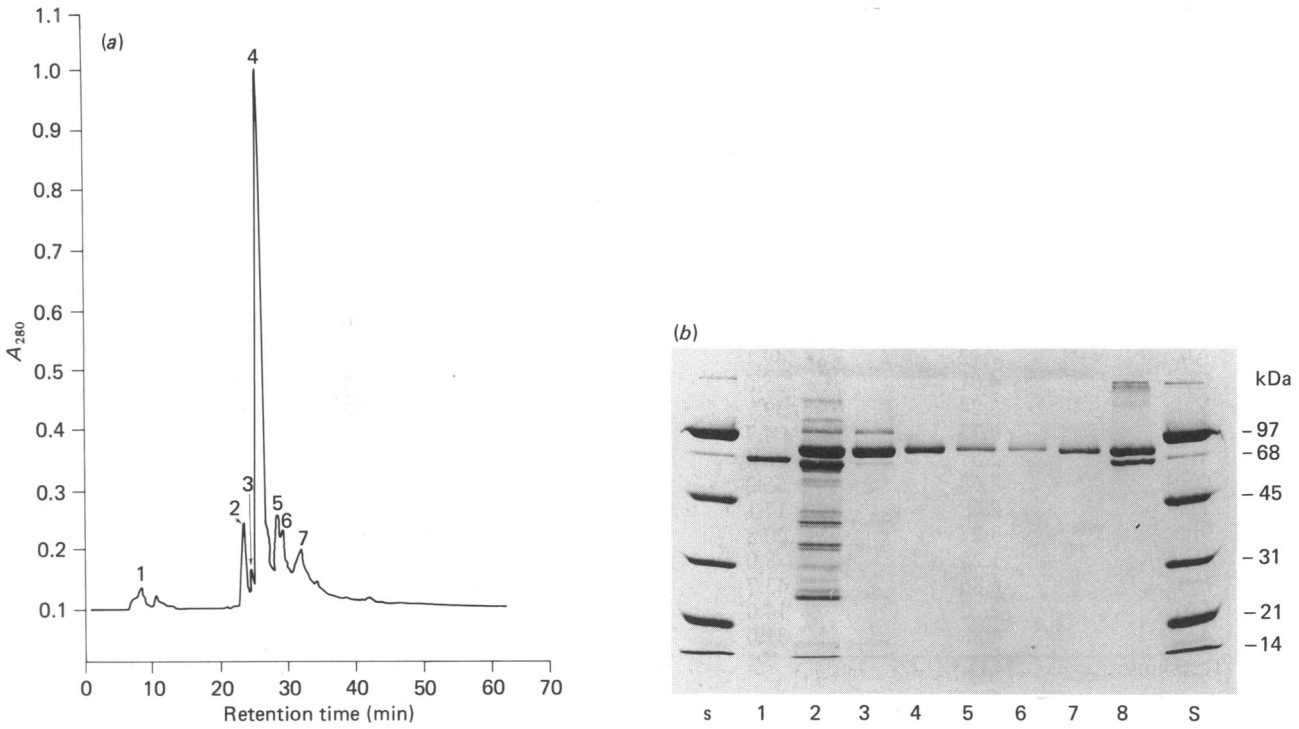

Fig. 3. Purification of the 70 kDa protein by reverse-phase h.p.l.c.

(*a*) Elution profile of proteins from the reverse-phase h.p.l.c. The flow was monitored at 280 nm. (*b*) SDS/polyacrylamide-gel-electrophoretic analysis of proteins from h.p.l.c. were individually collected and evaporated to dryness on a Speed Vac apparatus. Samples were redissolved in 10 mM-Tris/HCl buffer, pH 8.0. About 5–10 μg of protein was analysed by SDS/12%-polyacrylamide-gel electrophoresis. Lane 1, mouse albumin; lane 2, total protein of uterine luminal fluid; lane 3, protein eluted with 0.5 M-NaCl from CM-Affi-Gel Blue column chromatography; lanes 4, 5, 6 and 7, protein from h.p.l.c. corresponding to peaks 4, 5, 6 and 7 respectively; lane 8, equal amounts of albumin and h.p.l.c. peak 4 protein mixture. Molecular masses of marker proteins are indicated (lane S).

were desalted, freeze-dried and redissolved in a small amount of aq. trifluoroacetic acid 0.1% (starting h.p.l.c. buffer) and chromatographed on a reverse-phase h.p.l.c. column as described in the Experimental section. Fig. 3(*a*) shows the elution profile monitored at 280 nm. Under our chromatographic conditions, the major protein was eluted at 25 min (peak 4); several minor peaks were eluted between 27 to 32 min. The SDS/polyacrylamide-gel-electrophoretic analysis of the proteins from h.p.l.c. column peaks 4, 5, 6 and 7 are shown in Fig. 3(*b*). The peak 4 material yielded a single band at 70 kDa. The other peaks (5, 6 and 7) all have a major 70 kDa protein along with some minor contaminants at a lower-molecular-mass range. A mixture of 5 μg of the protein from h.p.l.c. peak 4 and an equal amount of purified mouse albumin (approx. 68 kDa) was resolved on SDS/polyacrylamide-gel electrophoresis with the mouse albumin running slightly faster than the 70 kDa protein (Fig. 3*b*, lane 8). This clearly demonstrates that the protein recovered from reverse-phase h.p.l.c. column has a molecular mass of approx. 70 kDa under SDS/polyacrylamide-gel-electrophoresis conditions. In order to determine whether the 70 kDa protein observed under the SDS/polyacrylamide-gel-electrophoresis conditions represents the size of the native protein in the uterine luminal fluid, we subjected uterine luminal fluid (1 ml) to gel filtration on Sephadex G-150. The fraction containing the peak level of the 70 kDa band by SDS/polyacrylamide-gel electrophoresis (without 2-mercaptoethanol) essentially corresponded to the elution peak for bovine albumin, determined by the absorbance at 280 nm.

Composition

Amino acid composition was determined after hydrolyzing the 70 kDa protein in 6 M-HCl for 20 h at 105 °C. No unusual amino acid composition was found (Table 1). A majority aspartic acid/asparagine and glutamic acid/glutamine residues must be present as amides to give an extremely basic protein without a large (Lys+Arg)/(Asx+Glx) ratio. Isoelectrofocusing showed that the 70 kDa protein is a basic protein that migrates between pI standards (broad pI calibration kit; Pharmacia) of 9.3 and 10 (results not shown). Analysis by the phenol/H_2SO_4 method revealed that 70 kDa protein contained 2.5–3.5% carbohydrate by dry weight. Individual sugar analysis yielded sialic acid, galactose, mannose, fucose and glucosamine (Table 2); the estimation of fucose content was higher than values for many *N*-linked glycoproteins (Kornfeld & Kornfeld, 1981; Bhattacharyya *et al.*, 1984).

Partial amino acid sequence

Automated Edman degradation of at least 1 nmol of the 70 kDa protein failed to reveal an *N*-terminal residue, which suggests that a blocked amino group may occupy

Table 1. Amino acid composition of the 70 kDa glycoprotein

Amino acid	Amino acid composition (Residues 100 residues)	(mol/mol of glycoprotein)*
Asx	9.74	61.9
Thr	5.87	37.3
Ser	7.18	45.7
Glx	11.56	73.6
Pro	6.16	39.2
Gly	8.32	52.9
Ala	7.72	49.1
Cys	4.01	25.6
Val	6.24	39.7
Met	0.73	4.7
Ile	2.57	16.4
Leu	8.68	55.2
Tyr	2.68	17.1
Phe	3.27	20.8
His	1.25	8.0
Lys	6.86	43.7
Trp	2.20	14.0
Arg	4.88	31.0
N-Terminus . . .	N.D.†	

* Based on a molecular mass of 70000 Da.
‡ Not detected.

Table 2. Carbohydrate composition of the 70 kDa glycoprotein

Residue	Carbohydrate composition (mol/mol of glycoprotein)
Sialic acid	1.81
Galactose	2.15
Mannose	3.0*
Fucose	1.84
Glucosamine	3.58

* Mannose set equal to 3.0.

this position. Partial sequence determination of the protein was then attempted on fragments obtained after cleavage with CNBr for 24 h. As shown in Fig. 4(*a*), separation of the cleavage products by h.p.l.c. revealed three major peaks when the eluate was monitored by 280 nm; a similar profile was observed at 214 nm. Re-chromatography of each of the peak fractions yielded peaks at the corresponding elution position. Automated Edman degradation of both peak 1 and peak 2 material indicated that two (or more) polypeptides were present. Further analysis of the peak proteins was made by SDS/polyacrylamide-gel electrophoresis. As shown in Fig. 4(*b*), the peak 1 material ran near the dye front (< 12 kDa). Peak 2 revealed a major band at approx. 30 kDa; other bands were observed at 38 and 26 kDa; some material also ran with the dye front. Peak 3 yielded a prominent band at approx. 40 kDa with minor components at 43 and 30 kDa. Automated Edman degradation of the peak 3 fraction (19 cycles) revealed the sequence given in Fig. 5. The sequence determined for peak 3 has been located in a cDNA for the mRNA of the 70 kDa protein, with complete homology except for residue 1, where the cDNA indicates phenylalanine rather than isoleucine, which is preceded by a methionine residue, as expected, in the translated cDNA (B. Pentecost & C. T. Teng, unpublished work). The partial amino acid sequence presented in Fig. 5 was screened through

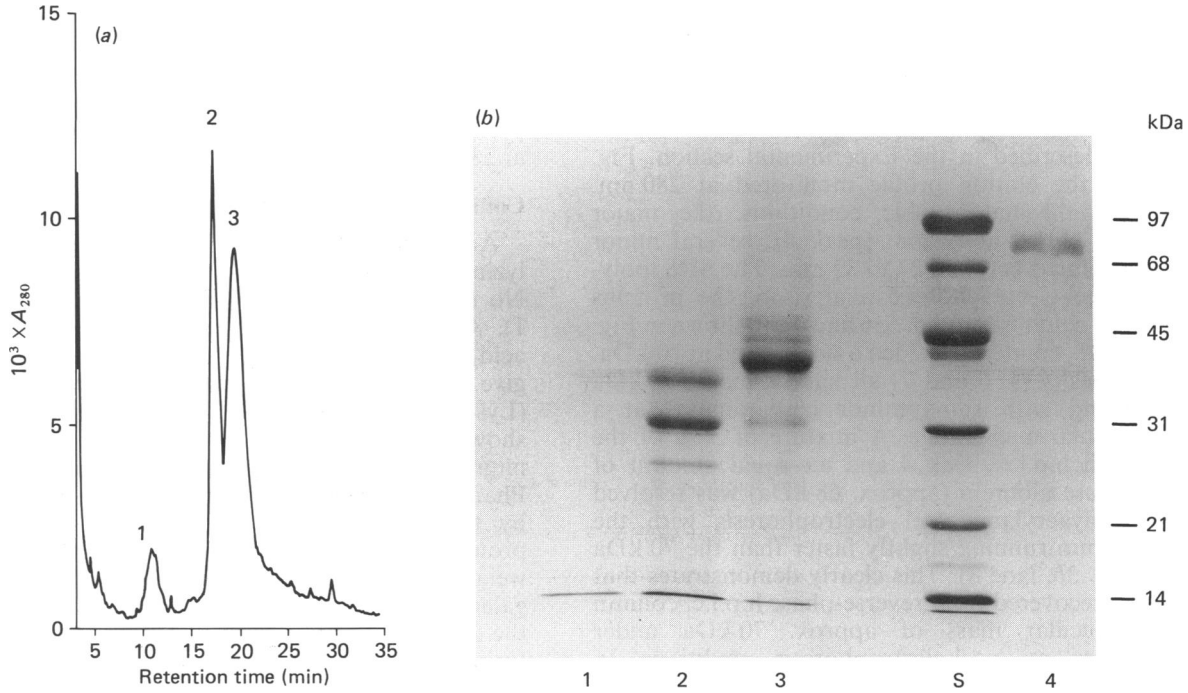

Fig. 4. Analysis of CNBr-cleaved 70 kDa protein

(*a*) Elution profile of the CNBr-cleaved 70 kDa protein from h.p.l.c. Fractions 1, 2 and 3 were individually collected and evaporated to dryness on a Speed Vac apparatus. The samples were analysed by SDS/polyacrylamide-gel electrophoresis (*b*). Lanes 1, 2 and 3 corresponding to peaks 1, 2 and 3. Lane 4, intact 70 kDa protein. Molecular masses of marker proteins are indicated (lane S).

Ile-Asp-Ala-Gly-Lys-Pro-Pro-Tyr-Lys-Leu-Arg-Pro-Val-Ala-Ala-Glu-Val-Tyr-Gly

Fig. 5. Amino acid sequence of a CNBr-cleaved fragment of the 70 kDa protein

The fragment corresponds to peak 3 material in the h.p.l.c. profile of Fig. 4(*a*).

Protein Sequence Data Bases of Georgetown University Medical Center. No significant sequence homologies with other proteins or glycoproteins were found. We have also randomly screened about ten shorter amino acid sequences (five to seven residues) through Protein Sequence Data Bases by using SCAN. Again, no sequence overlaps were found.

Antiserum production

Rabbit (8345) anti-(70 kDa protein) IgG yielded a single immunoprecipitation band by Ouchterlony immunodiffusion analysis when tested against the uterine luminal fluid, the CM-Affi-Gel Blue fraction (0.5 M-NaCl) and the h.p.l.c.-purified 70 kDa protein; there was no reactivity demonstrated with purified mouse albumin (approx. 68 kDa) or transferrin (approx. 76 kDa) (results not shown). Protein in the uterine luminal fluid from oestrogen-stimulated immature mice were separated on SDS/polyacrylamide-gel electrophoresis and blotted on to nitrocellulose paper (Fig. 6). India-ink staining of the blots demonstrated that almost all of the proteins were transferred to the nitrocellulose paper. After the blotting, the SDS/polyacrylamide gel was stained with Coomassie Blue to detect whether any protein still remained in the gel. It was found that the 70 kDa proten was the only protein that was not completely transferred to the nitrocellulose paper (results not shown). However, with immunoperoxidase staining of the blots, the 70 kDa protein band was the only protein stained.

Hormone specificity and tissue distribution

Fig. 7 shows the standard curve constructed with h.p.l.c.-purified 70 kDa protein by the e.l.i.s.a. The CM-Affi-Gel Blue fraction also produced a straight line with a slope similar to that for the 70 kDa protein. Quantitative determination of the 70 kDa protein in the uterine luminal fluid of an oestrogen-treated immature mouse has also been performed by e.l.i.s.a. It produced a straight line with a slightly different slope from that of the purified 70 kDa protein; the presence of non-specific binding components in the uterine luminal fluid may have contributed to such differences. The lower detection limit for the 70 kDa protein is 400 pg under the current assay conditions. This e.l.i.s.a. method was used to examine the tissue distribution and the potential induction of 70 kDa protein by various steroid hormones. No 70 kDa protein was detected in unstimulated, progesterone-treated or testosterone-treated immature mouse uteri; there was immunoreactive material, however, in mice receiving diethylstilboestrol for 3 days (Fig. 7). The amount of 70 kDa protein in the extract of uterine luminal content and tissue homogenate reaches to 1–2% of the total protein after oestrogen treatment.

Proteins from various tissues taken from an oestrogen-treated female mouse were also examined by using the e.l.i.s.a. Vaginal tissue was found to contain 70 kDa protein (about 10 ng/μg of protein). We did not detect any 70 kDa protein immunoreactivity in tissue extracts of liver, kidney, brain, spleen, lung, intestine or ovary. Seminal vesicles and the secretion of the gland from a male mouse were also negative for 70 kDa protein.

Proteinase and plasminogen activator activities were examined by SDS/polyacrylamide-gel electrophoresis on gels co-polymerized with gelatin and plasminogen. Proteinase activity was observed with uterine luminal fluid in the 80 kDa, 55 kDa and 25 kDa regions, and plasminogen activator activity was detected in the 35–45 kDa region of the gel. No proteinase or plasminogen activator activity was seen at 70 kDa region of the gel with uterine luminal fluid, partially purified 70 kDa protein in CM-Affi-Gel Blue fraction or h.p.l.c.-purified 70 kDa protein (results not shown).

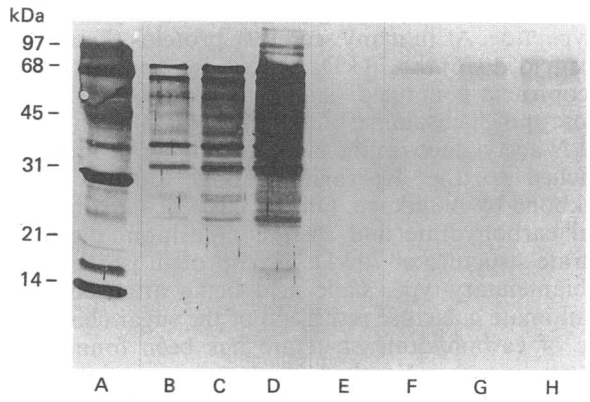

Fig. 6. Immunoblot of mouse uterine luminal fluid

Total uterine luminal fluid proteins (lanes B and F, 10 μg; lanes C and G, 25 μg; lanes D and H, 60 μg) and molecular-mass marker proteins (lanes A and E) were separated by SDS/12%-polyacrylamide-gel electrophoresis. The proteins were Western-blotted on to nitrocellulose paper as described in the Experimental section. Lanes A–D were stained with India ink; lanes E–H were immunostained with anti-(70 kDa protein) antibody.

DISCUSSION

Synthesis of uterine-specific proteins under the influence of steroid hormones has been demonstrated in the human (Sylvan *et al.*, 1981), rabbit (Nieto *et al.*, 1977), skunk (Fazleabas *et al.*, 1984) and rodent (Pinsker *et al.*, 1974; Surani, 1977; Kassis *et al.*, 1984; Komm *et al.*, 1985; Kuivanen & DeSombre, 1985). Attempts to isolate a hormone-responsive uterine-specific protein from uterine luminal fluid has been hampered by the presence of other serum proteins (Hirsch *et al.*, 1977; Voss & Beato, 1977; Oliphant *et al.*, 1978). CM-Affi-Gel Blue column chromatography provided a simple one-step separation of the uterine secretory 70 kDa protein from most of the luminal and serum proteins. Once the 70 kDa protein has been successfully separated from interference by serum proteins of similar molecular mass, it can be purified readily to homogeneity by a reversed-phase h.p.l.c. Uterine luminal fluid may contain proteins derived from Gram-positive and Gram-negative bacteria

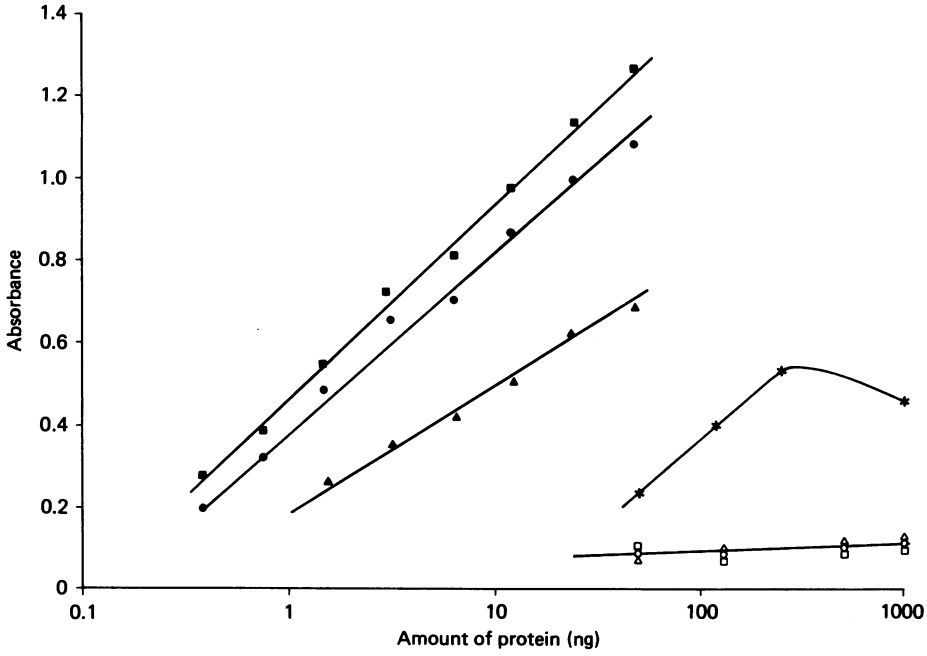

Fig. 7. Effect of steroid hormones on 70 kDa protein production

Various steroid hormones were administered to the immature mouse as described in the Experimental section. The uteri with the luminal content were homogenized in PBS and then centrifuged at 13000 *g* for 15 min. Total protein present in the soluble fraction was used for quantitative determination of 70 kDa protein by e.l.i.s.a. △, Unstimulated; ✱, diethylstilboestrol-treated; □, testosterone-treated; ○, progesterone-treated. Calibration curves: ■, h.p.l.c.-purified 70 kDa protein; ●, CM-Affi-Gel Blue (0.5 M-NaCl) fraction; ▲, uterine luminal fluid of oestrogen-treated immature mouse.

in addition to serum proteins. It has been reported that oestrogen treatment of intact or ovariectomized female rats allows the migration of bacteria of vaginal origin into the uterus, resulting in contamination of the uterine tissue and the accumulated fluid (Leland *et al.*, 1983). In order to minimize the possibility of bacterial contamination, we have collected uterine luminal fluid under sterile conditions from oestrogen-stimulated prepubertal mice. Furthermore, we have collected the uterine luminal fluid from oestrogen-stimulated germ-free mice as well, and analysed it by SDS/polyacrylamide-gel electrophoresis. These fluids have the same protein banding pattern as the uterine luminal fluid from conventionally housed immature mice treated with oestrogen (results not shown).

Fluorogram analysis of [^{35}S]methionine-labelled protein in oestrogen-stimulated incubation medium containing mouse uterine tissue indicated that the 70 kDa protein is one of the major proteins labelled in the incubation medium. In addition, we have detected the 70 kDa protein by Western-blot analysis in the conditioned medium of Percoll-purified uterine epithelial cells in culture from 26-day-old prepubertal mice (C. T. Teng, Y. Tomooka & J. A. McLachlan, unpublished work). It is possible that the 70 kDa protein may be rapidly secreted from the uterine luminal epithelial cells immediately after synthesis. It has also been observed that oestrogen stimulated the synthesis and release of proteins from uteri of the ovariectomized (Komm *et al.*, 1985) or immature rats (Kuivanen & DeSombre, 1985).

The 70 kDa protein was stimulated by oestrogen, but neither progesterone nor testosterone caused induction. We have also screened various tissues of an oestrogen-stimulated animal for 70 kDa protein: vaginal tissue was the only tissue other than uterus that gave a measurable amount of 70 kDa immunoreactivity. We could not exclude the possibility of contamination of vaginal tissue with uterine fluid.

Amino acid composition and partial sequence analysis suggest that the 70 kDa protein has not been previously reported. The combined data from SDS/polyacrylamide-gel electrophoresis and gel-filtration chromatography indicate that this protein is secreted as a single-chain polypeptide. As in many secretory proteins (Kornfeld & Kornfeld, 1976) the 70 kDa protein is glycosylated. This glycoprotein contained sialic acid, mannose, galactose, fucose and glucosamine, but no galactosamine, indicating that *N*-acetylglucosamine of the carbohydrate moieties is attached to the asparagine residue of the peptide backbone by *N*-linkage. On the basis of the amount of total carbohydrate and their composition, the carbohydrate structure of 70 kDa glycoprotein is likely to be of biantennary-type, sialic acid being attached to the penultimate galactose residue(s) of the sugar chain. This type of carbohydrate structure has been found to be present in many *N*-linked glycoproteins isolated from different sources (Kornfeld & Kornfeld, 1981).

In fact, the assembly of *N*-linked glycoporoteins in the mouse uterus has recently been shown to be stimulated by oestrogens (Dutt *et al.*, 1986). This oestrogen stimulation was antagonized by progesterone. These studies demonstrated that the oestrogen-stimulated glycoprotein synthesis in the uterus and its secretion was not due to increased enzyme activities for oligosaccharide assembly or transfer to protein, increased dolichyl phosphate availability or increased sugar nucleotide

availability, supporting the finding in the present study that oestrogen induces the synthesis of the 70 kDa glycoprotein rather than its processing. Thus the regulation of this gene should be of great interest.

The function of the 70 kDa protein is unknown. Judged from its biological and chemical characteristics it is probably distinct from other reported mammalian oestrogen-induced proteins such as rat uterine inducible protein (Notides & Gorski, 1966), peroxidase (Lyttle & DeSombre, 1977*a*,*b*), hydrolase (Katz *et al.*, 1980; Finlay *et al.*, 1982), 46 kDa protein secreted from MCF 7 cell line (Westley & Rochefort, 1979, 1980) and the progesterone receptor (Horwitz & McGuire, 1978). Peltz *et al.* (1983) have reported the presence of a 46 kDa and a 70 kDa plasminogen activator protein in rat uterus and uterine luminal fluid. After oestrogen stimulation, the plasminogen activator found in uterine luminal fluid increased 10-fold over the controls. We examined the plasminogen activator activity of oestrogen-stimulated mouse uterine luminal fluid and the purified 70 kDa protein, and found no evidence that the 70 kDa protein is the plasminogen activator.

The 70 kDa protein from the mouse uterus is strongly basic, glycosylated and secreted in abundance by the uterine epithelial cells after oestrogen stimulation. The apparent tissue- and hormone-specificity as well as the relative abundance of the uterine 70 kDa protein make it a good candidate for use as a marker of oestrogen-induced gene action in the mouse uterus. Our laboratory has been interested in the relationship between prenatal diethylstilboestrol exposure and subsequent genital-tract abnormalities (McLachlan *et al.*, 1980; Newbold *et al.*, 1984; Teng & Teng, 1985; Maier *et al.*, 1985); the possibility exists that the synthesis of the 70 kDa protein is affected by prenatal diethylstilboestrol exposure. On a more general level, the recent isolation of the corresponding gene (B. Pentecost & C. T. Teng, unpublished work) provides an opportunity to extend biochemical and pathological observations of these hormone-induced disturbances to the molecular level, since transient effects on gene methylation, appearance or disappearance of hypersensitive sites etc. might be detectable even in the absence of lasting effects and could provide an opportunity to define further the effects of oestrogenic hormone that cause subsequent abnormalities.

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